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Detection of *Toxoplasma gondii* in Raw Caprine, Ovine, Buffalo, Bovine, and Camel Milk Using Cell Cultivation, Cat Bioassay, Capture ELISA, and PCR Methods in Iran

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Abstract

This study was conducted to determine the presence of *Toxoplasma gondii* in animal milk samples in Iran. From a total of 395 dairy herds in three provinces of Iran, 66 bovine, 58 ovine, 54 caprine, 33 buffalo, and 30 camel herds were studied, and from these parts of Iran, 200 bovine, 185 ovine, 180 caprine, 164 buffalo, and 160 camel milk samples were collected from various seasons. Samples were tested for *Toxoplasma gondii* by cell line culture, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) technique. Only the results of cell line cultivation were confirmed by bioassay in cat. Results indicated that all herds were infected with *Toxoplasma gondii*. The culture method showed that 51 out of 889 milk samples (5.73%) were positive for *Toxoplasma gondii,* and all 51 positive culture results were positive with bioassay in cat. The Fars province had the highest prevalence of *Toxoplasma gondii* (6.84%). The ELISA test showed that 41 milk samples (4.61%) were positive for the presence of *Toxoplasma gondii,* while the PCR showed that 46 milk samples were positive for *Toxoplasma gondii*. The results showed higher sensitivity of PCR and higher specificity of ELISA. Caprine had the highest (10%) and camel had the lowest (3.12%) prevalence rate of parasite. The summer season had the highest (76.47%) but winter (3.92) had the lowest incidence of *Toxoplasma gondii*. This study is the first prevalence report of direct detection of *Toxoplasma gondii* in animal milk samples in Iran.

Introduction

Milk is raised as a complete food, especially for children and seniors. Its high value for proteins, minerals, fats, and vitamins is undeniable, and on any given day, millions of people eat milk and dairy products. Therefore, the hygienic quality of milk has a high importance in public health, but sometimes infections and illness occur. Toxoplasmosis is the most widespread zoonotic disease worldwide, caused by an obligate intracellular protozoan parasite called *Toxoplasma gondii*, which can infect warm blood vertebrates, including mammals and birds species throughout the world. Toxoplasmosis in humans may occur vertically by tachyzoites that are passed to the fetus via the placenta, or horizontally, which may involve three life-cycle stages: (1) ingesting sporulated oocysts from cats, (2) ingesting tissue cysts in raw or under cooked meat, or (3) ingesting tachyzoites in blood products or primary offal (viscera) of many different animals, organ transplants, and unpasteurized milk (Tenter *et al*., 2000).

Several studies have shown that consumption of infected milk can cause disease in human (Acha and Szyfres, 2003; Manal *et al*., 2006; Camossi *et al*., 2011). The milk of affected animals contains tachyzoites, which facilitates transmission to offspring. Among the studies on horizontal transmission, only a few have reported the presence of tachyzoites in the milk of different species, including sheep, goats, camel, and cattle (Dubey, 1998; Tenter *et al*., 2000; Manal *et al*., 2006). Based on geographic location, 15–85% of the human population is asymptomatically infected with *T. gondii* (Reisch *et al*., 2003). It is estimated that more than 60 million people in the United States carry the *Toxoplasma* parasite, with 30–50% of the U.S. population having antibodies to *T. gondii,* and at least 30% of people worldwide are infected with the organism (Aspinall *et al*., 2002), with a higher prevalence of infection in France, where 60–90% of the population are serologically

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positive (Ajioka *et al*, 2001). In addition, *T. gondii* is the third leading cause of foodborne death in the United States (Gagne, 2001). Toxoplasmosis can cause a large range of clinical manifestations in humans, from abortion and congenital infection to eye disease (when acute infection occurs in seronegative pregnant women) and fetal encephalitis in chronically infected immunocompromised hosts such as patients with advanced HIV infection (Al-Qurashi *et al*., 2001; Marcinek *et al*., 2008; Kamani *et al*., 2010).

Current diagnosis of *T. gondii* is based on serological detection, cell culture, bioassays, and molecular methods (Switaj *et al*., 2005). Polymerase chain reaction (PCR) is a major breakthrough for the diagnosis of *T. gondii* infection (Martins *et al*., 2000). Several studies have shown the higher accuracy, sensitivity, and specificity of PCR technique than traditional diagnostic methods (Held *et al*., 2000; Kompalic-Cristo *et al*., 2007).

The epidemiology and prevalence of *T. gondii* in raw milk samples is essentially unknown, and this present study was performed for the detection of *T. gondii* in raw bovine, ovine, caprine, buffalo, and camel milk samples in Iran.

Materials and Methods

Samples collection

In order to study several risk factors (origin of milk samples collected and various seasons) to determine the prevalence of *T. gondii*, from January 2011 to January 2012 (in various seasons of the year) a total of 200 bovine, 185 ovine, 180 caprine, 164 buffalo, and 160 camel milk samples (250 mL of each animal) were collected randomly from three major provinces of Iran (Tehran, Isfahan, and Fars). There were 100 bovine, 92 ovine, 88 caprine, 60 buffalo, and 55 camel herds in these areas, and we chose 66 bovine, 58 ovine, 54 caprine, 33 buffalo, and 30 camel herds from these regions (Table 1). The animals from which milk samples were collected for this study were clinically healthy, and the milk samples showed normal physical characteristics. Samples were collected under sterile hygienic conditions and were immediately transported at 4°C to the laboratory in a cool box with ice packs. All milk samples were kept at −20°C until processing.

Milk preparation and cell line cultivation

Milk samples (200 mL) were homogenized and passed through a 3m/N filter (Sartorius Ltd., Gottingen, Germany), to remove cells, and washed at 1300 × *g* for 15 min four or five times with sterile phosphate-buffered saline (PBS). Next, tachyzoites of *T. gondii* were enumerated under fluorescence by acridine orange propidium iodide staining on a hemocytometer and numbers were routinely determined by phase-contrast microscopy (Olympus); then, tachyzoites were suspended in modified Eagle's medium, at a concentration of 103 tachyzoites/mL, for challenge in cell lines. African green monkey cells (Vero) were grown in a 75-cm^3 tissue culture flask (Corning, High Wycombe, UK) in modified Eagle's medium (1×). This was supplemented with 4 mM of L-glutamine (Bio-Whittaker, UK), gentamicin (40,000 U; Roussell Laboratories, Uxbridge, UK), fungizone (1 mg; Squibb and Sons, Hounslow, UK), 50 μg/mL streptomycin (Sigma, St. Louis, MO), 50 U/mL penicillin (Sigma), 4,500 mg/L glucose, 110 mg/L sodium pyruvate, and fetal bovine serum (FCS) at a concentration of 10%, and incubated at 37°C in 5% CO_2 until there was a completely confluent monolayer of cells. The cells were then transferred into a 2% FCS maintenance medium and incubated at 37°C in 5% CO_2 for 24 h.

Cat bioassay

The positive results of the cell line culture (51 milk samples) were tested by bioassay in cat. All cats used in the present study were 10–12 weeks old, had been raised in isolation from birth (Pasteur Institute of Iran), and had not been fed uncooked meat before these experiments. Blood was drawn from a jugular vein of each cat 1 week before feeding milk, and the sera were tested for antibodies to *T. gondii* using the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). No demonstrable MAT antibodies were found in a 1:25 dilution of the sera. After testing all cats, 50 mL of milk from each animal species was fed individually to 51 cats over a 2–3-day period. Each cat was housed individually, and the feces were collected daily for 14 days beginning 3 days after feeding of milk. Feces were microscopically examined for oocysts by fecal flotation (Dubey, 1995).

Capture Enzyme-Linked Immunosorbent Assay (ELISA)

All milk samples were tested for *T. gondii* with capture ELISA test. Multilevel plates (Nunc) were absorbed with polyclonal rabbit antiserum to *T. gondii* concentration of 30 μg/mL in coating buffer (PBS, pH 7.2). After overnight incubation at 4°C and washing, the samples (milk) were added to each well and held for 1 h at 37°C. The plates were washed three times with PBS with Tween-20 (PBST). Rabbit anti–*T. gondii* immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) were diluted 1:10, in PBST, added to each well, and held for 1 h at 37°C. After washing, the chromogenic substrate orthophenylen-diamidine (Sigma–

Table 1. Distribution of Bovine, Ovine, Caprine, Buffalo, and Camel Herds in Tehran, Isfahan, and Fars Provinces of Iran, and Numbers of Selected Herds for This Study

	No. herds in the study region					*No. herds studied*					*No. samples per herd*				
Provinces	*Bovine*	*Ovine*	*Caprine*	*Buffalo*	*Camel*	*Bovine*	*Ovine*	*Caprine*	*Buffalo*	*Camel*	*Bovine*	*Ovine*	*Caprine*	*Buffalo*	*Camel*
Tehran	40	37	28	8	5	33	26	12	5	2	4–10	2–8	3–9	4–6	4–8
Isfahan	30	30	20	22	41	15	16	14	10	22	8–15	5–12	6–12	5–8	9–10
Fars	35	25	40	30	9	18	16	28	18	6	10–19	7–16	7–15	5–10	6–10
Total	100	92	88	60	55	66	58	54	33	30	4–19	2–16	3–15	4–10	4–10

Aldrich, St. Louis, MO) was added to each well. After incubating for 20 min, the enzymatic activity was revealed. The reaction was stopped by addition of sulfuric acid 20%. Absorbance was recorded at 492 nm as detected with an automated ELISA reader (Foroghiparvar *et al*., 2008).

DNA extraction and PCR

DNA was isolated from raw milk samples using the Qiagen QIAamp System (Qiagen, Valencia, CA), which specifically binds nucleic acids to a silica-gel membrane in a centrifugecompatible spin column. Contaminants and PCR inhibitors were washed through, and DNA was eluted using 70°C water. A sample volume of 200 μL was used per extraction. All milk samples were tested for *T. gondii* with PCR technique (Burg *et al*., 1989). A 193-bp (base pair) product of the 35-fold repetitive B1 gene of the RH strain of *T. gondii* was amplified using the following primers: TX2 5′-3′ (TCT TTA AAG CGT TCG TGG TC) and TX4 5′-3′ (GGA ACT GCA TCC GTT CAT GAG) (Burg *et al*., 1989). The amplification reactions were performed using Amplitaq DNA polymerase, GeneAmp dNTPs (deoxynucleoside triphosphates) with dUTP, and AmpErase UNG (all from Perkin Elmer, Foster City, CA). The PCR-amplified products were examined by electrophoresis in a 1.5% agarose gel, stained with a 1% solution of ethidium bromide, and examined under ultraviolet illumination. In this study, *T. gondii* DNA and DNase-free water were used as the positive and negative controls, respectively.

Ethical issues

All cats used in experiments were handled using procedures approved by the Animal Care Program of the U.S. Department of Agriculture. After testing, all infected cats were treatment with clindamycin (25–50 mg/kg/day) (divided into two or four doses), which continued for several days.

Statistical analysis

Statistical analysis was performed using SPSS/18.0 software for assessing a significant relationship between hot and cold seasons for occurrence of parasite in milk samples. Chi-square test was performed, and differences were considered significant at a p-value of < 0.05.

Results

A total of 889 animal milk samples were tested by cell line culture, capture ELISA, and PCR methods, and only positive cell line culture results were tested with bioassay in cat. The results of four diagnostic assays are shown in Tables 2 and 3. Our results showed that all 66 bovine, 58 ovine, 54 caprine, 33 buffalo, and 30 camel selected dairy herds were infected with *T. gondii,* but the prevalence rate in milk samples was low. On the other hand, 100% of selected herds were infected with this parasite. Using the culture method, 51 of 315 milk samples (5.73%), including 17 in Tehran (5.39%), 14 in Isfahan (4.96%), and 20 in Fars (6.84%) provinces, were found to be contaminated with *T. gondii*. All 51 positive cell line cultures had positive cat bioassay too. The *T. gondii* antigen was detected in 41 (4.61%) milk samples, and only 10 samples were diagnosed as negative by ELISA test. After PCR, the B1 gene of the RH strain of *T. gondii* was detected in 46 (5.17%) milk samples. On the other hand, for the total 51 positive milk samples, ELISA and PCR methods detected 41 and 46 samples, respectively.

Table 2. Detection and Study of the Distribution of *Toxoplasma gondii* by Cell Line Cultivation, Bioassay in Cat, Capture ELISA, and PCR Methods in Three Major Provinces of Iran

Provinces	*No. milk samples*	*Cell line cultivation (%)*	*Bioassay in cat (%)*	*Capture ELISA (%)*	*PCR (%)*
Tehran	315	17 (5.39)	17 (5.39)	13 (4.12)	15 (4.76)
Isfahan	282	14 (4.96)	14 (4.96)	11 (3.9)	13 (4.6)
Fars	292	20 (6.84)	20 (6.84)	17 (5.82)	18 (6.16)
Total	889	51 (5.73)	51 (5.73)	41 (4.61)	46 (5.17)

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

Using the cell line cultivation as the gold standard (i.e., assuming the cell line cultivation detected 100% of positive samples), the detection methods ranked as follows, in descending order of sensitivity, PCR (test sensitivity 90.1%) > capture ELISA (test sensitivity 80.3%), and in descending order of specificity, capture ELISA (test specificity 98.5%) > PCR (test specificity 96.4%%) (Table 4).

In this study, bioassay in cat only confirmed the cell line culture results. On the other hand, the oocyst of *T. gondii* was detected in feces of all 51 cats. Results showed that the Fars province (6.84%) had the highest and the Isfahan province (4.96%) had the lowest prevalence of *T. gondii* in raw milk in Iran. The prevalence of *T. gondii* in caprine was the highest (10%), while the prevalence of *T. gondii* in camel was the lowest (3.12) in the positive samples. Our results revealed that the milk samples that were collected in summer (76.47%) followed by those collected in the spring (11.76%) had the highest and the milk samples collected in winter (3.92%) followed by those collected in autumn (5.88%) had the lowest prevalence of *T. gondii*, respectively (Table 5). Our results indicated significant differences ($p < 0.05$) in the level of contamination with *T. gondii* between milk samples collected from different species and $p < 0.05$ between the level of milk contamination with *T. gondii* in summer with other seasons. Significant differences were seen ($p < 0.05$) between cell line culture and ELISA test. There were no significant differences between the ELISA and PCR methods.

Table 3. Detection of *Toxoplasma gondii* in Bovine, Ovine, Caprine, Buffalo, and Camel Milk Samples by Cell Line Cultivation, Bioassay in Cat, Capture ELISA, and PCR Methods

Species	*No. milk samples*	*Cell line cultivation (%)*	*Bioassay in cat (%)*	*Capture ELISA (%)*	*PCR (%)*
Bovine	200	8 (4)	8 (4)	6 (3)	7 (3.5)
Ovine	185	13 (7.02)	13 (7.02)	11 (5.94)	12 (6.48)
Caprine	180	18 (10)	18 (10)	16 (8.88)	17 (9.44)
Buffalo	164	7 (4.26)	7 (4.26)	5 (3.04)	6 (3.65)
Camel	160	5 (3.12)	5 (3.12)	3 (1.87)	4 (2.5)
total	889	51 (5.73)	51 (5.73)	41 (4.61)	46 (5.17)

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

Table 4. Evaluation of Sensitivity and Specificity of ELISA (A) and PCR (B) Techniques for Detection of *Toxoplasma gondii* in Milk Samples

(A)

	Cell line culture (+)	*Cell line culture (−)*	*Total*
ELISA (+)	$41^{*,a}$	12^{c}	53
ELISA (−)	10^{b}	$826^{**,d}$	836
	51^{a+b}	838^{c+d}	889

*Sensitivity: $\frac{a}{a+b}$=80.3%.

**Specificity: $\frac{d}{d+c}$=98.5%.

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

(B)

	Cell line culture (+)	*Cell line culture (−)*	*Total*
PCR (+)	$46^{*,a}$	30^{c}	76
PCR (−)	5^{b}	$808^{**,d}$	813
	51^{a+b}	838^{c+d}	889

*Sensitivity: $\frac{a}{a+b}$=90.1%.

**Specificity: $\frac{d}{d+c}$=96.4%.

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

Discussion

Our results showed that milk can easily be contaminated with tachyzoites of *T. gondii* and it is not specific to a particular species. However, caprine and ovine had a highest prevalence of *T. gondii* in milk samples but bovine, buffalo and camel species can also shed tachyzoites from their milks. This finding is important in terms of public health especially in the case of cow's milk which has a highest human consumption. Even in some area of Iran consumption of raw milk is common. Although it has not been considered but our results revealed that all dairy herds in these regions were infected with *T. gondii* and probably consumption of contaminated raw milk can cause infection in human on these areas. The results of our study showed that the high significant differences between the presence of *T. gondii* in bovine, ovine, caprine, camel and buffalo milk samples. In addition, the levels of contamination in summer had significant differences with other seasons especially winter season. Therefore, the levels of milk contamination with *T. gondii* are more significant in the warm seasons of the year. Besides, the seasons, origin of milks and animal species all are the risk factors for occurrence of *T. gondii*.

Table 5. Distribution of *Toxoplasma gondii* in Bovine, Ovine, Caprine, Buffalo, and Camel Milk Samples in Various Seasons of the Year

Species	*No. of positive samples with culture*	*Spring (%)*	*Summer (%)*	*Autumn (%)*	*Winter (%)*
Bovine	8	1 (12.5)	5 (62.5)	1 (12.5)	1 (12.5)
Ovine	13	2 (15.38)	11 (84.61)	—	—
Caprine	18	2 (11.11)	14 (77.77)	1 (5.55)	1
Buffalo	7	1 (14.28)	4 (57.14)	1 (14.28)	1 (14.28)
Camel	5	—	5 (100)	—	—
Total	51	6 (11.76)	39 (76.47)	3 (5.88)	2 (3.92)

Therefore, our results showed that in cases when we do not have much time to perform diagnostic methods like cell line culture and cat bioassay, using ELISA or PCR as an accurate, specific, sensitive, rapid and even safe diagnostic methods, are not unpleasant. Cell line culture and cat bioassay both are accurate techniques but they are associated with a high risk of laboratory-acquired infections and very time consuming and in cases of cat bioassay treatment of infected cats is expensive. Bovine, buffalo and camel are not the primary hosts of *T. gondii* and the high prevalence of this parasite in bovine, buffalo and camel species maybe showed the close contact of these animals with infected cats.

The high prevalence of *T. gondii* in summer season showed that this parasite needs proper temperature (warm weather) to complete its life cycle. Besides, some other transmission routes of *T. gondii* have been considered, and previous studies have raised the possibility of transmission by blood-sucking arthropods, in particular, ticks (Sroka *et al*., 2003; Sroka *et al*., 2008). Previous study from Iran showed that *Ixodes ricinus* which is a major tick for transmission of *T. gondii*, was found in Iran (Bashiribod *et al*., 2004). After analyzing the relative humidity of Fars, Isfahan and Tehran provinces (63%, 28%, and 39%, respectively) it was recognized that the prevalence rate of *T. gondii* in these provinces is related with their relative humidity. On the other hand, the ticks need high humidity for their living and in Fars province the average relative humidity on January 2011 to January 2012 was higher than Tehran and Isfahan. Information showed that the relative temperature of summer in this area of Iran was 40°C on average, while in autumn, winter, and spring average temperatures were 15°C, 6°C, and 19°C. Our results showed significant differences ($p<0.05$) between the relative humidity of Fars with Tehran and Isfahan provinces, and $p<0.05$ between relative temperature of summer with autumn, winter, and spring. Therefore, the higher prevalence of *T. gondii* in Fars province is due to higher relative humidity of this province, and the higher prevalence of *T. gondii* in summer season is due to higher relative temperature of this season. In addition, the relative humidity in warmer seasons is higher than colder seasons. So, the higher temperature and humidity provide appropriate conditions for ticks and they can easily transmitted disease to their hosts. The prevalence of this protozoon in animal milk samples is higher in summer season; thus, inspection and control of milk for the presence of *Toxoplasma* is essential in this season.

The high prevalence of *T. gondii* infection in ovine and caprine may be due to the association of free-range sheep livestock with *T. gondii* infection. They are kept on pastures and have an increased pressure of infection due to contamination of the environment with oocysts. In the United States, the frequency of stray cats in a humid rainy climate, which favors the survival of oocysts, has contributed to high *Toxoplasma* prevalence (Remington *et al*., 2001). In Iran, stray cats are widespread in Tehran, Isfahan, and Fars provinces, and a higher prevalence of oocysts in the humid environment there and farming animal rearing is also common. To our knowledge, eating undercooked sheep or goat meat, drinking raw

sheep or goat milk, and preparation of raw sheep or goat meat were the risk factors that had the strongest influence on acquiring toxoplasmosis. However, any raw meat exposure or drinking any raw milk (of various animals) had less influence, followed by own exposure or exposure to cats. Previous research showed that the milk of several species of animals (including sheep, goats, cows, and mice) was contaminated and could serve as a source of toxoplasmosis in humans (Powell *et al*, 2001), in agreement with our results. Our results, for the first time, showed that bovine, buffalo, and camel species can shed *T. gondii* into their milk, and the prevalence of parasite in buffalo milk samples (4.26%) was higher than in two other species (4% and 3.12%, respectively).

A few years after the first reports of human infection after milk consumption (Riemann *et al*., 1975; Sacks *et al*., 1982; Skinner *et al*., 1990), most researchers agreed that milk can be a source of human toxoplasmosis (Dubey and Beattie, 1988; Powell *et al*., 2001); however, Dubey (1986, 1994) reported that milk from infected cows is regarded as being of negligible importance because cattle are resistant to *T. gondii* infection (Huong *et al*., 1998); however, our results showed that milk from infected bovine, ovine, caprine, buffalo, and camel is a source of *T. gondii* and can be easily transferred to humans. Our results are in agreement with Skinner *et al*. (1990), who suggested that more attention should be given to milk as well as meat from goats as a potential source for human toxoplasmosis because of their greater susceptibility to infection and their higher rate of seropositivity than cattle (Gondim *et al*., 1999).

Previous research showed that routine serologic diagnosis of toxoplasmosis provides high sensitivity, but specificity varies depending on the test used (Liesenfeld *et al*., 1996), while our results showed that the ELISA test had high specificity (98.5%) but its sensitivity was low (80.3%).

Our results showed that small ruminants and especially caprine had a highest prevalence of *T. gondii* (10%). This finding is in agreement with results reported by Tenter *et al*. (2000), who reported that small ruminants showed high seroprevalences in many areas of the world up to 92%.

In another study, *T. gondii* DNA was detected in seven milk samples from five seropositive sheep and twice in milk of two sheep. Sequences of species shared 97–100% identity with *T. gondii* (Camossi *et al*., 2011). These recent findings suggested the hypothesis that the peripartum period may also lead to the resurgence of bradyzoites in tissue cysts into the active and rapidly replicating tachyzoite stage which can circulate again and be excreted in the milk. Another study on experimentally infected camels (Manal *et al*., 2006) showed that these antibody-positive camels shed *Toxoplasma* tachyzoites in their milk, but the number of *Toxoplasma* tachyzoites excreted in the milk of female camels was low, and this is in agreement with our results.

To our best knowledge, this present study is the first prevalence report of direct detection of *T. gondii* in bovine, ovine, caprine, buffalo, and camel milk samples using cell line cultivation, cat bioassay, ELISA, and PCR techniques in Iran.

Conclusion

We conclude with several general observations. We recommend, first, close inspection and control of milk and especially caprine milk in those area or seasons that have warm and humid weather; second, proper application of PCR or ELISA techniques to provide an accurate, safe, sensitive, specific, and fast diagnostic method that can be used to monitor the parasitical load in milk; third, the use of pasteurized milk, which is well controlled; fourth, since milk contamination in Iran has a seasonal pattern, improved hygiene in warm seasons of the year; and fifth, if possible, all staff in milk factories should be serologically negative for *T. gondii*.

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Disclosure Statement

No competing financial interests exist.

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